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Regulation of NF-κB, Th Activation, and Autoinflammation by the Forkhead Transcription Factor Foxo3a

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Summary

Forkhead (Fox) transcription factors play key roles in immunoregulation. Members of the Foxo subfamily have been implicated in the regulation of the cell cycle and/or apoptosis, but their specific immunological contexts remain largely undefined. We demonstrate here that Foxo3a, the predominant Foxo member expressed in peripheral lymphoid organs, plays a critical role in lymphoid homeostasis. Foxo3a deficiency leads to spontaneous lymphoproliferation, associated with inflammation of several organs, in the absence of overt apoptotic defects. These findings correlated with the presence of hyperactivated helper T cells, which proliferated more vigorously and produced more Th1 and Th2 cytokines than their wild-type counterparts. Foxo3a inhibits NF-κB activation, whose overactivity was responsible for T cell hyperactivity in Foxo3a-deficient mice. Thus, Foxo3a regulates helper T cell activation and tolerance by inhibiting NF-κB activity, reinforcing a generalized role for the forkhead proteins in the maintenance of T cell tolerance through the inhibition of inflammatory transcriptional activities.

Introduction

The Foxo subfamily of forkhead transcription factors includes at least four members, Foxo1 (Fkhr), Foxo3a (Foxo3, Fkhrl1), Foxo4 (Afx), and Foxo6 (Jacobs et al., 2003), which have been implicated in the regulation of the cell cycle and apoptosis (Birkenkamp and Coffer, 2003; Tran et al., 2003). In the absence of environmental signals or growth factors, the Foxo family members remain transcriptionally active in the nucleus. Relevant growth factors activate the phosphatidylinositol 3-kinase (PI3K)-Akt (protein kinase B, PKB) signaling pathway, phosphorylating the Foxo proteins and rendering them susceptible to 14-3-3-assisted nuclear export, abrogating transcriptional activity (Tran et al., 2003). In this way, the PI3K-Akt-Foxo pathway has been proposed to modulate diverse biological processes, including cell survival, proliferation, apoptosis, and stress responses via target genes including GADD45, Cdkn1a/p21, Cdkn1b/p27, cyclin B, and Bim (Birkenkamp and Coffer, 2003; Brunet et al., 2004; Motta et al., 2004; Tran et al., 2003).

The Foxo genes are the mammalian homologs of *Caenorhabditis elegans* DAF-16, which regulates insulin signaling and metabolism as well as the control of organismal life span and fertility. Gene targeting in mice has demonstrated that Foxo1 regulates both insulin sensitivity (Kitamura et al., 2002; Nakae et al., 2002) and adipocyte differentiation (Nakae et al., 2003), while Foxo3a regulates ovarian development and fertility (Castrillon et al., 2003; Hosaka et al., 2004).

In the immune system, the Foxo members have been strongly suggested to regulate lymphoid homeostasis (Birkenkamp and Coffer, 2003). In the human leukemia T cell line Jurkat, Foxo1 overexpression suppresses proliferation (Medema et al., 2000), while Foxo3a overexpression induces apoptosis (Brunet et al., 1999). Similarly, Foxo3a overexpression in the murine pre-B cell line Ba/F3 can also induce apoptosis (Dijkers et al., 2002), and in the murine CTLL-2 T cell line, IL-2, via PI3K, leads to the inhibition of Foxo family members, which regulate the cell cycle inhibitor Cdkn1b (p27), the proapoptotic Bcl family member Bim (Stahl et al., 2002), and the antiapoptotic gene glucocorticoid-induced leucine zipper (GILZ; Asselin et al., 2004). Thus, the Foxo's may regulate lymphocyte quiescence (Coffer, 2003; Yusuf and Fruman, 2003); however, these studies have primarily been confined to transformed or cultured lymphocyte cell lines, rather than primary cells.

Nonetheless, a clinically relevant immunoregulatory role for the Foxo family members remains likely, since a growing number of forkhead genes are being demonstrated to regulate T cell activation, homeostasis, and tolerance: Foxp3, for example, has received attention for its role in regulatory $CD4^+CD25^+$ T cells (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003), and Foxn1 is required for T cell development due to its role in thymic epithelium (Frank et al., 1999). In addition, attention to Foxj1 has developed in our laboratory during studies in systemic lupus erythematosus (SLE), a systemic autoimmune syndrome characterized by the development of pathogenic autoantibodies, such as anti-DNA, which mediate lethal end-organ disease, such as glomerulonephritis (Mills, 1994). Foxj1 expression is significantly diminished in lymphocytes from lupus-prone mouse strains, which recapitulate the immune-complex pathogenesis of the human disease, but not nonautoimmune strains (Andrews et al., 1978; Lin et al., 2004). Foxj1-deficient T cells demonstrate accentuated proliferation and cytokine responses to antigen receptor and mitogenic signaling, leading to autoreactivity and multisystem organ inflammation resembling the cellular autoimmunity of lupus. These effects reflect a requirement for Foxj1 to suppress spontaneous NF-κB activity in naive T cells, likely via the activation of IκB proteins (Lin et al., 2004). Since hyperactivity of NF-κB has been implicated in the pathogenesis of multiple autoimmune syndromes, including SLE (Peng, 2004), an elucidation of the mechanisms by which the forkhead transcription factors and/or other immunoregulatory genes regulate NF-κB activity will likely provide insight into the pathogenesis of autoimmunity in general.

During ongoing studies, we have found that Foxo3a, like Foxj1, can also inhibit NF-κB activation, suggesting

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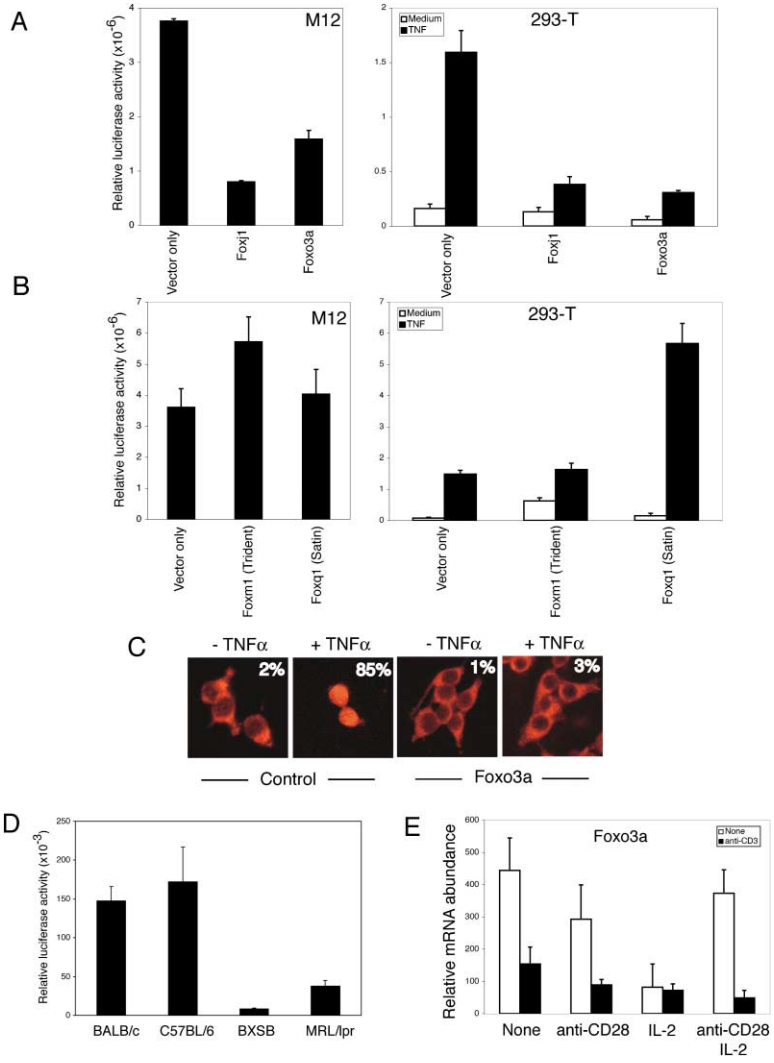

Figure 1. Foxo3a Is an NF-κB Antagonist

(A) Spontaneous activity of an NF-κB-luciferase reporter in M12 cells was assessed in the presence of control (pcDNA), Foxj1-expressing (pcDNA-Foxj1), or Foxo3a-expressing (pcDNA-Foxo3a) expression vectors. Inducibility of an NF-κB-luciferase reporter construct was assessed in 293T cells in the presence (black bars) or absence (open bars) of TNF-α, in the presence of control (pcDNA), Foxj1-expressing (pcDNA-Foxj1), or Foxo3a-expressing (pcDNA-Foxo3a) expression vectors.

(B) Similar studies were performed in M12 and 293T cells in the presence of control, Foxm1-, or Foxq1-expressing vectors.

(C) Nuclear translocation capabilities of RELA in 293T cells was assessed in the presence or absence of Foxo3a. After retroviral tranduction with a control (pMX-IRES-GFP) or Foxo3a-expressing (pMX-Foxo3a-IRES-GFP) retrovirus, cells were stimulated with TNF-α for 45 min and stained for RELA by immunohistochemistry (red). All cells shown were GFP positive (green fluorescence not shown). Percentages indicate the number of cells, per 100 GFP-positive cells, that demonstrated nuclear RELA staining.

(D) Spontaneous Foxo activity was assessed in naive Th cells from nonautoimmune (BALB/c, C57BL/6) versus lupus-prone (BXSB, MRL/lpr) mice using a 6XDBE-luciferase reporter.

(E) Naive $CD4^+$ T cells were isolated from wild-type C57BL/6 mice and incubated in the presence (black bars) or absence (open bars) of 1 μg/ml plate bound anti-CD3, as well as, where indicated, soluble anti-CD28 and/or recombinant IL-2. After 24 hr, expression of Foxo3a was determined by real-time PCR. Error bars indicate the standard deviations of three simultaneously performed samples, representative of three experiments.

that Foxo3a may have similar immunoregulatory functions in vivo. Indeed, in the absence of functional Foxo3a, mice develop a spontaneous, multisystemic inflammatory syndrome associated with lymphadenopathy, increased NF-κB activation, and hyperactivation of $CD4^+$ T cells, similar to Foxj1 deficiency. Thus, Foxo3a also regulates autoimmunity and helper T cell activation, suggesting a general role for the forkhead genes in the control of lymphocyte tolerance.

Results and Discussion

Identification of Foxo3a as an NF-κB Antagonist with Depressed Activity in Autoimmunity

During ongoing studies with Foxj1, we wondered if NF-κB repression was a unique characteristic of Foxj1, and we tested the ability of several forkhead members to inhibit NF-κB activity in vitro. Intriguingly, Foxo3a, but not Foxm1 or Foxq1, could suppress NF-κB activity in both M12 and 293T cells (Figures 1A and 1B). It consistently was not as effective as Foxj1 in M12 cells in suppressing spontaneous NF-κB activity ($p < 0.10$, comparing luciferase activity in the presence of Foxj1 versus Foxo3a) but was consistently comparable to, if not more effective than, Foxj1 in 293T cells (note the lower luciferase activity in the presence of Foxo3a versus Foxj1, whether or not treated with TNF). Foxo3a overexpression could also inhibit TNF-induced nuclear translocation of the RELA NF-κB subunit in 293T cells (Figure 1C; $p < 0.001$, comparing Foxo3a to control). Thus, like Foxj1, Foxo3a is an NF-κB antagonist.

Since the in vivo NF-κB hyperactivity that results from Foxj1 deficiency may contribute to the T cell hyperactivity and tolerance loss in autoimmune diseases like SLE (Lin et al., 2004), Foxo3a deficiency might likewise contribute to autoimmunity by allowing NF-κB hyperactivity and T cell autoactivation. To explore this possibility, we first assessed endogenous Foxo activity in naive T cells purified from nonautoimmune and lupus-prone animals at 5–6 weeks of age, several weeks before the onset of clinically apparent disease (Andrews et al., 1978). As judged by a Foxo-luciferase reporter, Foxo activity was significantly lower in Th cells from lupus-prone, compared to nonautoimmune, mice (Figure 1D; $p < 0.0001$, comparing either BXSB or MRL/lpr to BALB/c or C57BL/6). Since Foxo3a is the dominant Foxo member expressed

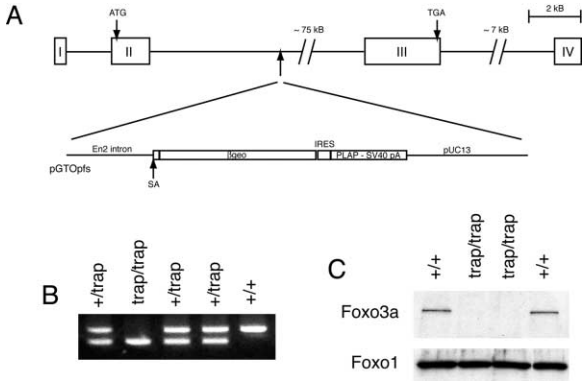

Figure 2. Targeting of the Murine Foxo3a Locus

(A) Genomic organization of murine Foxo3a, with the insertion site of the gene-trap vector in BayGenomics ES cell line XA026, as determined by our laboratory, indicated by an arrow. Roman numerals indicate exon numbers. The start (ATG) and stop (TGA) sites of the coding exons are indicated.

(B) PCR-based genotyping of the Foxo3a trapped allele. Tail DNA from mice of the indicated genotypes was amplified by standard PCR.

(C) Western blotting of thymi from 4-week-old +/+ or trap/trap mice was performed using a polyclonal antibody raised against the whole Foxo3a molecule. Foxo1-specific antibodies were used as a loading control. En2, engrailed 2; SA, splice acceptor site.

in peripheral lymphoid organs (Biggs et al., 2001; Furuyama et al., 2000), these findings suggest that loss of function of Foxo3a might indeed be responsible at least in part for NF-κB hyperactivation in these lupus-prone mice. Interestingly, the RNA expression and activity pattern of Foxo3a in conventional, nonautoimmune Th cells is highly reminiscent of Foxj1, being highly expressed and active in naive Th cells and diminishing rapidly upon anti-CD3 and/or IL-2 stimulation, also supporting this notion (Figure 1E; Lin et al., 2004; Stahl et al., 2002). Thus, as an NF-κB antagonist with depressed activity in Th cells from lupus-prone mice and a predominant expression in naive Th cells, Foxo3a might exert an anti-inflammatory function in T cells in vivo.

Autoinflammation and Lymphoproliferation in the Absence of Foxo3a

To test this, we derived animals deficient in Foxo3a by generating animals bearing a disabled Foxo3a allele using the BayGenomics XA026 embryonic stem cell line, which was derived during a gene-trap targeting strategy (Figure 2). Our genetic analysis of the cell line determined the gene-trap insertion site to be located ~5 kb downstream of the first coding exon, such that the strong splice acceptor (SA) site of the insert would result in aberrant splicing of the transcript (Figure 2A). Germline transmission of this allele was achieved (Figure 2B), and intercross of heterozygotic animals yielded +/+, +/trap, and trap/trap animals of approximately Mendelian ratios. When examined by Western blot, Foxo3a was undetectable in thymi of trap/trap animals (Figure 2C). Thus, the targeted mutation of XA026 appears to be a null allele. However, although we have been unable to detect mature mRNA corresponding to exons 2–3 of the Foxo3a gene in trap/trap thymi (not shown), we cannot rule out the possibility that finite levels of mature, wild-type Foxo3a may form as a result of alternative splicing around the targeting vector. In addition, although we have failed to detect truncated forms of Foxo3a that might correspond to exon 2 using antibodies that recognize the form of Foxo3a phosphorylated at Thr-32 (data not shown), we cannot rule out the possibility that finite levels of such a truncated Foxo3a, undetectable by our assays, are also produced. Therefore, we refer to the genotype of these animals as trap/trap.

Trap/trap animals appeared outwardly healthy until at least 8 months of age, although females were infertile after 12 weeks of age due to abnormal ovarian follicular development, consistent with previously described Foxo3a-targeted alleles (Castrillon et al., 2003; Hosaka et al., 2004). Histopathological examination of older (>16 week) animals revealed that they suffered from multisystem inflammation, particularly of the salivary gland, lung, and kidney (Figure 3; $p < 0.0001$, comparing +/+ to trap/trap for involvement of any organ shown). Immunohistochemical examination revealed a mixed infiltrate of $CD4^+$ and $B220^+$ cells, suggesting a lymphoid, presumably T-dependent, infiltrate. In addition, lymphoproliferation was evident, with splenic and lymph node sizes and cellularities typically 1.75–2 times their +/+ littermates (Figures 4A and 4B; $p < 0.01$, comparing cellularity of trap/trap to +/+ for either spleen or lymph node). This lymphadenopathy did not reflect a generalized organomegaly, since trap/trap animals developed hearts, kidneys, thymi, and livers of weights comparable to their +/+ counterparts (22.3 ± 1.6 versus 22.4 ± 1.5 mg, 23.0 ± 1.0 versus 23.9 ± 0.9 mg, 39.3 ± 1.4 mg versus 39.0 ± 1.0 mg, and 1.54 ± 0.16 versus 1.53 ± 0.13 g, respectively; $n = 5$ of each genotype). The lymphadenopathy was composed almost entirely of T cells, as evidenced by flow cytometric analyses of these organs, with B cell numbers remaining largely constant between +/+ and trap counterparts (Table 1; Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/21/2/203/DC1). Interestingly, the CD4/CD8 ratio of T cells remained largely normal, with approximately 2–3 times as many CD4 as CD8 cells in all organs tested of both +/+ and trap/trap animals. Nonetheless, trap/trap $CD4^+$ T cells appeared somewhat activated, with modest upregulation of CD44 and CD25, as well as downregulation of CD45RB, compared to their +/+ littermates. These findings were unlikely to reflect a role for Foxo3a in the development of regulatory T cells, since the $CD25^+CD62L^+CD4^+$ population was, if anything, proportionally increased in trap/trap animals. In addition, trap/trap animals did not demonstrate significant hypergammaglobulinemia or autoantibody production, as judged by tests for serum Ig, antinuclear antibodies, rheumatoid factor or anti-dsDNA, or gross abnormalities in B cell subsets (Table 1; Supplemental Figure S1, and data not shown), suggesting that the inflammatory phenotype predominantly reflected T, not B, cell hyperactivity.

Th Hyperactivation and Normal Apoptosis in the Absence of Foxo3a

Given the prior implications for Foxo3a in the regulation of apoptosis (Birkenkamp and Coffer, 2003; Coffer, 2003), an immediately obvious explanation for the in-

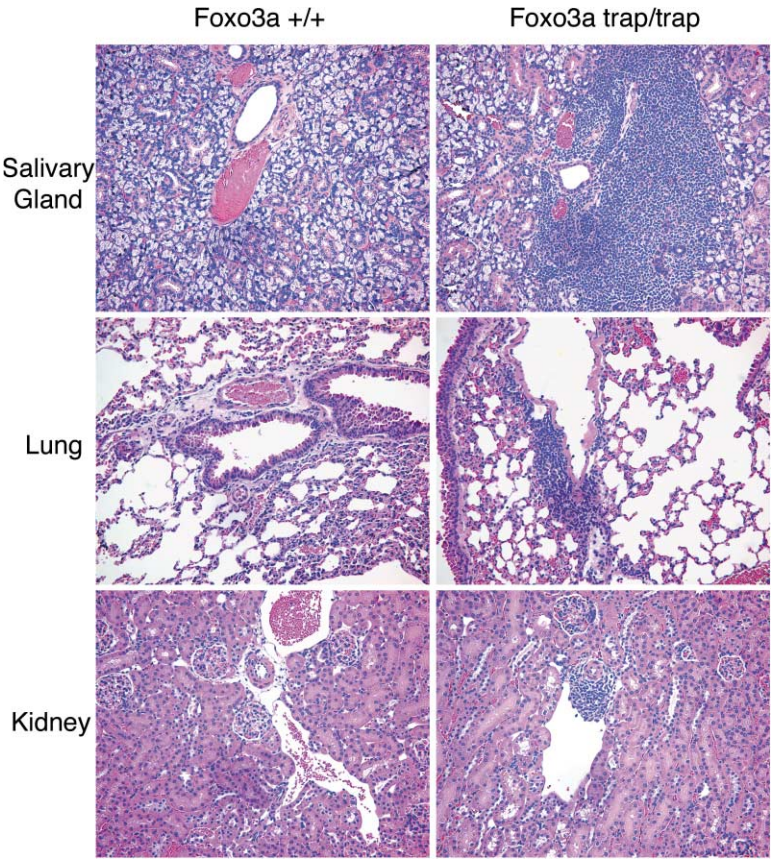

Figure 3. Autoimmune Inflammation in the Absence of Foxo3a

Note the heterogeneous inflammation of the organs in Foxo3a trap/trap animals, in contrast to normal-appearing tissues in +/+ counterparts. Each panel is from a different animal, representative of 10 animals examined for each genotype at 20 weeks of age. Original magnification, 20× for all panels.

flammatory and lymphoproliferative phenotype in trap/trap mice involves defects in activation-induced cell death or other apoptotic phenomena in T cells, reminiscent of the autoimmune and lymphoproliferative phenotype of animals with defects in CD95 or CD178 (Cohen and Eisenberg, 1991). However, trap/trap T cells were not impaired in their ability to undergo apoptosis, as judged by assays for activation-induced cell death; IL-2 withdrawal; anti-CD3-mediated deletion of $CD4^+CD8^+$ thymocytes in vivo; anti-CD95-induced lethal multisystem apoptosis in vivo; and anti-CD95, PMA, or ionomycin-mediated apoptosis in vitro (data not shown). As such, we conclude that Foxo3a is largely dispensable for immunoregulation by apoptosis.

As a result, lymphoproliferation in the absence of Foxo3a must instead have reflected defects in the regulation of T cell proliferation and/or activation. Indeed, bulk $CD4^+$ T cells from trap/trap mice proliferated much more vigorously than their +/+ counterparts, requiring only IL-2 in the absence of anti-CD3 or anti-CD28 for significant proliferation (Figure 4C; $p < 0.001$, comparing trap/trap to +/+, for all conditions tested without anti-CD28, for all anti-CD3 concentrations also stimulated with anti-CD28 and IL-2, and for all anti-CD3 concentrations $\geq$1 μg/ml also stimulated with anti-CD28). Similar results were observed when percentages of BrdU-positive cells were assessed by flow cytometry (e.g., 0.23 ± 0.08 versus 0.21 ± 0.08, 0.2 ± 0.03 versus 14.0 ± 1.0, 7.7 ± 1.0 versus 17.6 ± 1.9, and 20.7 ± 3.8 versus 44.0 ± 4.2, comparing +/+ to trap/trap Th cells stimulated with no stimulus, IL-2 alone, anti-CD3 alone, or anti-CD3 and IL-2, respectively; $p < 0.001$ for all conditions except no stimulus; $n = 3$ for each genotype).

Naive $CD4^+$ trap/trap T cells generally also proliferated better than their +/+ counterparts (Figure 5A; $p < 0.001$, comparing trap/trap to +/+, for 10 μg/ml anti-CD3 alone and 1 and 3 μg/ml anti-CD3 supplemented with IL-2), but these differences were modest compared to the differences seen with bulk $CD4^+$ T cells, suggesting that a significant proportion of trap/trap T cells undergo primary stimulation in vivo, presumably to autologous antigens (Lin et al., 2004). Indeed, trap/trap, but not +/+, T cells proliferate vigorously in autologous mixed lymphocyte reactions (Figure 4D), suggesting strongly that autoreactive trap/trap T cells are activated in vivo.

All the same, naive trap/trap $CD4^+$ T cells were clearly more hyperactivatable than their +/+ counterparts, as evidenced by significantly increased IL-2 production ($p < 0.001$ at all conditions tested with anti-CD3 > 1 μg/ml) and IFN-γ production ($p < 0.001$ at all conditions tested with anti-CD3 $\geq$ 3 μg/ml supplemented with anti-CD28 ± IL-2; Figure 5B). Similarly, under Th2-polarizing conditions, trap/trap Th cells consistently produced increased amounts of the Th2 cytokines IL-4, IL-5, IL-6, and IL-10 (Figure 5C; $p < 0.001$ at all conditions tested with anti-CD3 > 1 μg/ml). Interestingly, trap/trap $CD4^+$ cells exhibited less dramatic differences from their +/+ counterparts for both IL-2 and IFN-γ production when stimulated in the presence of IL-2 (Figure 5B; e.g., compare cells stimulated with anti-CD3 and IL-2 without anti-CD28), consistent with the ability of IL-2 to induce

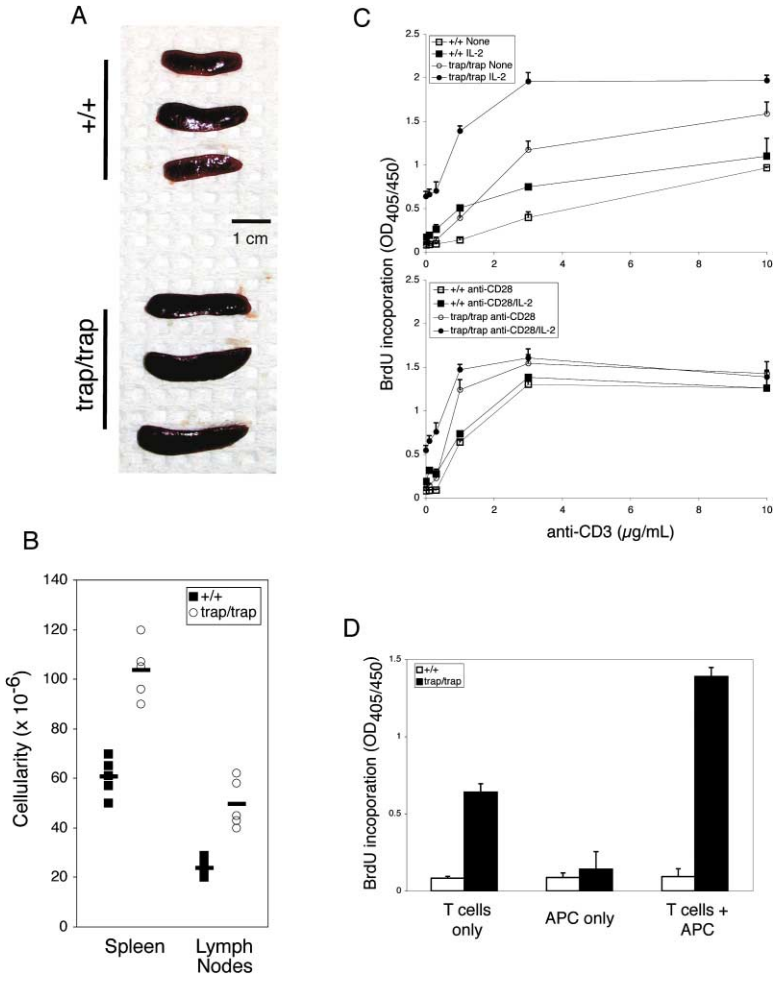

Figure 4. Lymphadenopathy, T Cell Hyperproliferation, and Autoreactivity in the Absence of Foxo3a

(A) Gross photomicrographs of spleens from 8-week-old +/+ or trap/trap mice.

(B) Cellularity of spleens and lymph nodes from 8-week-old +/+ or trap/trap mice (n = 5 in each group).

(C) Proliferative capacity of bulk $CD4^+$ T cells from Foxo3a +/+ (squares) and trap/trap (circles) animals, stimulated with the indicated amounts of plate bound anti-CD3 in the presence (black) or absence (open) of IL-2, with (lower panel) or without (upper panel) anti-CD28.

(D) Autologous mixed lymphocyte reactivity of $CD4^+$ T cells from Foxo3a +/+ (open bars) or trap/trap (black bars) mice. Th cells were incubated in the presence or absence of autologous splenic antigen presenting cells (APC) and assessed for proliferation by BrdU incorporation on day 3. Both +/+ and trap/trap cells proliferated vigorously (>OD 2.0) to concanavalin A (not shown). Error bars represent standard deviations of cells from three individual mice, representative of three independent experiments.

a Foxo3a deficiency by both suppressing RNA synthesis and inactivating the mature protein (Figure 1E; Stahl et al., 2002). Foxo3a deficiency thus leads to dysregulated Th cytokine production, hyperactivation, hyperproliferation, and inflammation, in part by mimicking IL-2 stimulation.

Role of NF-κB Activation in the Absence of Foxo3a

Based upon the parallel findings between Foxj1, Foxo3a, and NF-κB (Figure 1; Lin et al., 2004), NF-κB hyperactivation should be present and account for the hyperactive phenotype of trap/trap animals. Indeed, as judged by an NF-κB-luciferase reporter, naive trap/trap $CD4^+$ T cells demonstrated higher spontaneous NF-κB, but not NF-AT, activity compared to their +/+ counterparts (Figure 6A; $p < 0.001$) and blockade of NF-κB activity via double-stranded decoy NF-κB oligonucleotides, encoding the consensus binding site for all NF-κB subunits, effectively abrogated the hyperactivity, which was best evidenced by IL-2 overproduction (Figures 5B and 6B; $p < 0.0001$, comparing T cells treated with decoy or control decoy oligonucleotides). Thus, trap/trap T cells demonstrate NF-κB hyperactivation, which accounts for the T cell hyperfunction.

Given the role of Foxo3a as a transcription factor, a likely explanation for NF-κB hyperactivation in trap/trap animals would reflect its role in the regulation of inhibitory NF-κB proteins, such as the IκB family members (Li and Verma, 2002). Naive trap/trap Th cells contained significantly less IκBβ and IκBε, but not IκBα, RELA, or p50, mRNA than their +/+ counterparts ($p < 0.01$, Figure 6C), and demonstrated significantly less IκBβ and IκBε protein by Western analysis (Figure 6D), consistent with this possibility. However, the importance of Foxo3a in the regulation of IκBβ and IκBε transcription may be indirect: an examination of the putative promoter regions of these genes reveals no Foxo binding sites (TTGTTTAC), and Foxo3a fails to transactivate either an IκBβ- or IκBε-promoter-luciferase construct in vitro (Budde et al., 2002; Lin et al., 2004; and our unpublished data). Interestingly, trap/trap T cells also contained significantly less Foxj1 RNA than their +/+ counterparts (Figure 6C), suggesting that Foxo3a may actually regulate IκB transcription via Foxj1 (Lin et al., 2004); however, Foxj1 primary regulates IκBβ, not IκBε (Lin et al., 2004), so Foxo3a likely regulates the IκB's by additional mechanisms. Regardless, these findings together demonstrate that Foxo3a prevents autoimmunity and lymphoproliferation by suppressing inflammatory transcriptional activities like NF-κB, perhaps via other anti-inflammatory transcriptional regulators like Foxj1, but nevertheless ultimately via the IκB proteins.

Table 1. Cellularity of Lymphoid Organs in the Absence of Foxo3a

Genotype	Organ	Total ($\times 10^{-6}$)	CD3$^+$ (%)	B220$^+$ (%)	CD4$^+$ (% of CD3$^+$)	CD8 (% of CD3$^+$)	Calculated Number ($\times 10^{-6}$) CD3$^+$	B220$^+$	CD4$^+$	CD8$^+$
+/+	Spleen	50	41	58	71	27	21	29	15	5.5
+/+	Spleen	70	45	55	69	30	32	39	22	9.5
+/+	Spleen	61	39	60	73	26	24	37	17	6.2
+/+	Spleen	65	42	58	72	26	27	38	20	7.1
+/+	Spleen	57	41	59	74	25	23	34	17	5.8
	Avg	61	42	58	72	27	25	35	18	6.8
	Stdev	7.6	2.2	1.9	1.9	1.9	4.2	3.9	2.7	1.6
+/+	L. Node	20	74	25	69	30	15	5.0	10	4.4
+/+	L. Node	24	78	22	73	27	19	5.3	14	5.1
+/+	L. Node	21	79	20	73	26	17	4.2	12	4.3
+/+	L. Node	26	72	28	72	28	19	7.3	13	5.2
+/+	L. Node	29	71	27	70	29	21	7.8	14	6.0
	Avg	24	75	24	71	28	18	5.9	13	5.0
	Stdev	3.7	3.6	3.4	1.8	1.6	2.2	1.6	1.7	0.7
trap/trap	Spleen	96	63	36	70	29	60	35	42	18
trap/trap	Spleen	90	68	32	68	30	61	29	42	18
trap/trap	Spleen	105	60	39	69	30	63	41	43	19
trap/trap	Spleen	107	62	37	73	26	66	40	48	17
trap/trap	Spleen	120	61	39	72	27	73	47	53	20
	Avg	104	63	37	70	28	65	38	46	18
	Stdev	11.5	3.1	2.9	2.1	1.8	5.2	6.8	4.7	1.0
trap/trap	L. Node	45	87	10	72	27	39	4.5	28	11
trap/trap	L. Node	58	86	13	73	27	50	7.5	36	13
trap/trap	L. Node	40	89	11	69	31	36	4.4	25	11
trap/trap	L. Node	43	84	15	70	30	36	6.5	25	11
trap/trap	L. Node	62	90	9.8	76	25	56	6.1	42	14
	Avg	50	87	12	72	28	43	5.8	31	12
	Stdev	9.8	2.4	2.2	2.7	2.4	9.1	1.3	7.8	1.6

Spleens and lymph nodes (L. Node) of animals were assessed at 10–12 weeks of age using flow cytometry. Averages (Avg) and standard deviations (Stdev) are shown.

The Role of Foxo3a in the Regulation of T Cell Activation

Along with findings regarding Foxj1 (Lin et al., 2004), the present findings suggest a general role for forkhead transcription factors in the intrinsic enforcement of T cell quiescence and/or tolerance. The phenotype of Foxo3a deficiency is highly reminiscent of Foxj1 deficiency, including multisystem end-organ inflammation as well as T cell and NF-κB hyperactivation. Nonetheless, there are clear differences between Foxj1 and Foxo3a: whereas Foxj1 deficiency led to a severe, essentially lethal inflammatory syndrome affecting multiple vital organs, at least in chimeric animals using irradiated recipients (Lin et al., 2004), Foxo3a deficiency here in spontaneous, native animals leads to a mild, apparently nonlethal inflammation, predominantly affecting the salivary gland. In addition, Foxj1 deficiency led to Th1 hyperactivation in vivo, whereas Foxo3a deficiency here resulted in global hyperactivation of both Th1 and Th2 cells, and while Foxj1's NF-κB antagonist activity could be attributed solely to its regulation of IκBβ, Foxo3a regulates at least both IκBβ and IκBϵ. These differences likely account for the partly overlapping but still independent anti-inflammatory pathways regulated by both Foxo3a and Foxj1 in vivo, resulting in the differences in target organ involvement, Th1 versus Th2 cytokine production, etc. As such, it will be of interest to compare the activity of Foxj1 to Foxo3a in nonirradiated animals, such as through the use of a Foxj1 conditional allele, and/or to determine the relative importance of the various IκB family members to specific autoimmune syndromes, beyond their generally accepted roles as generic anti-inflammatory agents (Li and Verma, 2002).

Despite substantial suggestions in the literature (Tran et al., 2003), Foxo3a appears to be largely dispensable for the induction of apoptosis or activation-induced cell death in T cells. These results do not entirely rule out the possibility that Foxo3a regulates T cell apoptosis in certain contexts but suggest that its major function in vivo is antiproliferative or antiactivation by regulating anti-inflammatory or antiactivation targets like the IκB proteins. Alternatively, Foxo3a may regulate cell cycle proteins, like p27kip1 (Chandramohan et al., 2004; Medema et al., 2000), but the absence of spontaneous proliferation by naive trap/trap T cells argues against this possibility (Figure 5). At the same time, Foxo3a itself can be regulated by IκB kinases (Hu et al., 2004), suggesting a regulatory network comprised of the NF-κB and Foxo systems: on one hand, the IκB kinases can amplify NF-κB activity by initiating the degradation of both IκB proteins and Foxo3a. On the other hand, cessation of IκB kinase activity can result in Foxo3a reactivation and subsequent induction of IκB gene transcription, inhibiting NF-κB activity. Thus, Foxo3 likely modulates

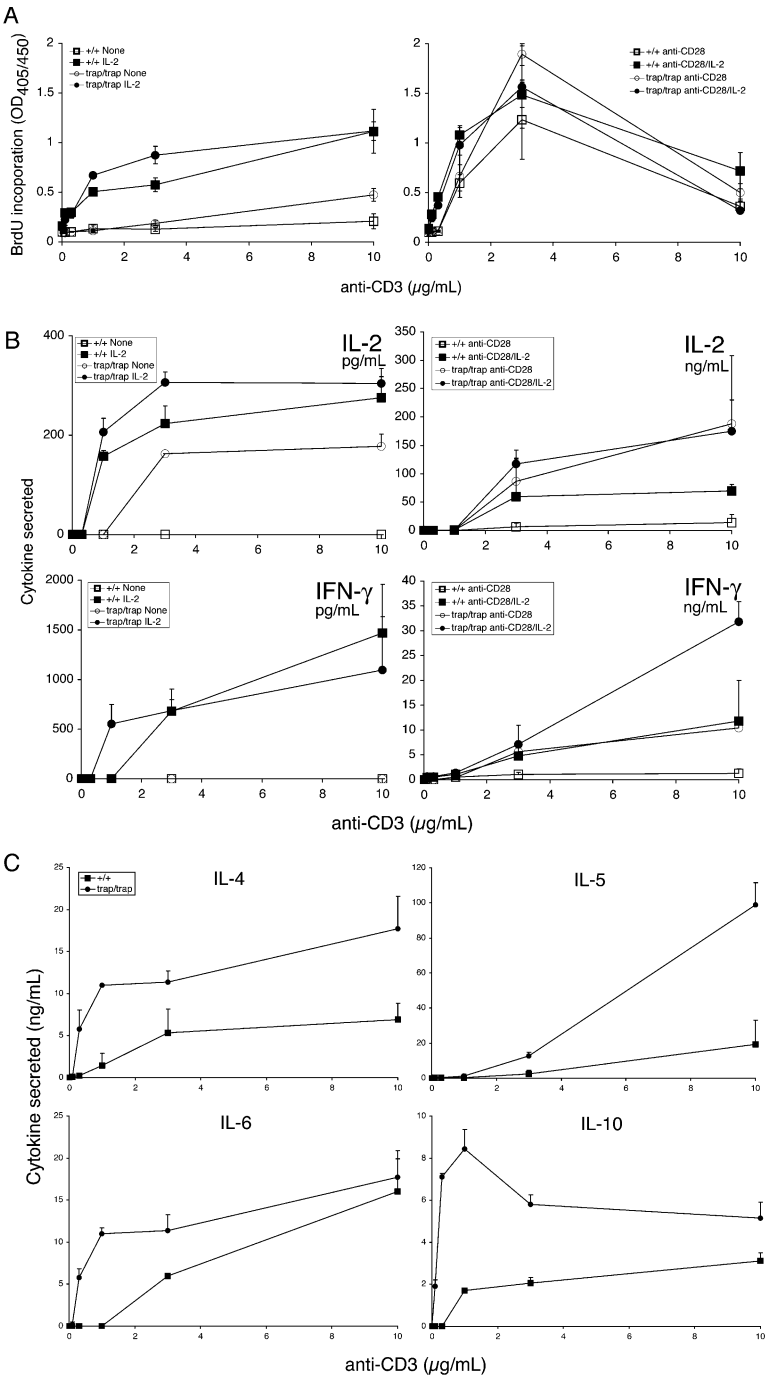

Figure 5. Hyperactivation of T Cells in the Absence of Foxo3a

(A) Proliferative capacity of naive $CD4^+$ T cells from Foxo3a +/+ (squares) and trap/trap (circles) animals, stimulated with the indicated amounts of plate bound anti-CD3 in the presence (black) or absence (open) of IL-2, with (right panel) or without (left panel) anti-CD28.

(B) Secreted cytokines produced by naive $CD4^+$ T cells from Foxo3a +/+ (squares) versus trap/trap (circles) animals, stimulated with the indicated amounts of plate bound anti-CD3 in the presence (black) or absence (open) of IL-2, with (right panel) or without (left panel) anti-CD28.

(C) Naive T cells from Foxo3a +/+ (squares) versus trap/trap (circles) animals were differentiated under Th2 polarizing conditions in vitro for 1 week, followed by stimulation with the indicated doses of anti-CD3. After 20 hr, cytokine secretion was assessed by ELISA. Standard deviations reflect triplicate samples, representative of four separately performed experiments.

activation thresholds as part of a closely coordinated signaling network in the Th cell, effectively reducing cell metabolism while maintaining the resting, naive cell state.

Recent studies on different Foxo3a deficiencies did not report the immunological abnormalities, including autoinflammation, described here (Castrillon et al., 2003; Hosaka et al., 2004). However, those analyses did not specifically address the immune system, and histological analyses of organs were performed at 9 weeks of age, well before the 16–20 weeks of age at which the trap/trap mice of the present study exhibited disease. Still, it will be of interest to compare the immunological phenotypes of these various Foxo3a alleles, particularly in older animals, to determine if the autoinflammatory syndrome reflects differences in environment and/or genetics.

Finally, the present findings suggest that loss of function in Foxo3a, other Foxo family members, and/or their respective metabolic pathways may contribute to the pathogenesis of autoimmune diseases by lowering the threshold for activation and tolerance loss. However, since isolated loss of function of Foxo3a fails to cause as severe an inflammatory phenotype as inbred lupus-

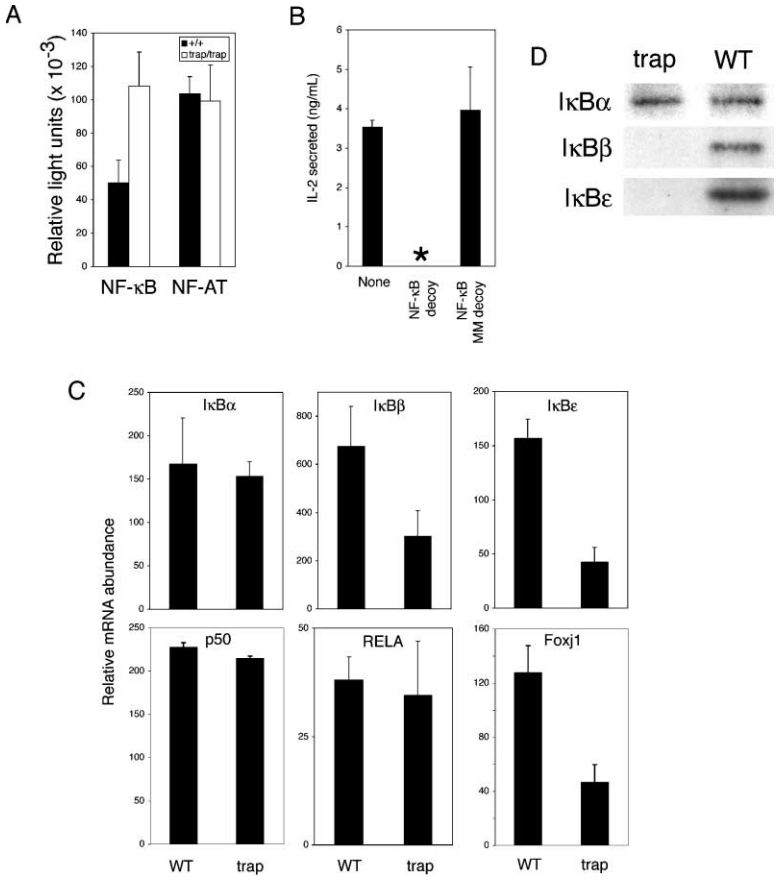

Figure 6. NF-κB Overactivation in the Absence of Foxo3a

(A) NF-κB- and NF-AT-luciferase activity was assessed in primary $CD4^+$ T cells derived from Foxo3a +/+ versus trap/trap animals.

(B) Naive T cells from Foxo3a trap/trap animals were stimulated in vitro with plate bound anti-CD3 (3 μg/ml) in the presence or absence of decoy NF-κB or control mismatch (MM) oligonucleotides. On day 3, IL-2 secretion was evaluated by ELISA on culture supernatants. Under these conditions, wild-type T cells fail to produce significant levels of IL-2 (Figure 5 and not shown).

(C) Levels of IκB, RELA, p50, and Foxj1 mRNAs were assessed by real-time PCR in naive Th cells from Foxo3a +/+ versus trap/trap animals.

(D) Levels of IκB proteins were assessed by Western blot in naive Th cells from Foxo3a +/+ (wt) versus trap/trap animals. Error bars indicate the standard deviations of three simultaneously performed samples, representative of two experiments.

prone mice, multiple polygenetic defects are likely required to culminate in an extreme, disease-inducing NF-κB hyperactivity. Such considerations suggest the potential importance of an examination of lymphoid systems multiply deficient for Foxo3a, Foxj1, and/or other transcription factors that also inhibit NF-κB yet are diminished in activity in autoimmune conditions. Such continued investigation will undoubtedly shed further insight into the mechanisms of T cell tolerance, quiescence, and activation.

Experimental Procedures

Mice

129, BALB/c, BXSB/MpJ, MRL/MpJ-CD95$^{lpr/lpr}$ (MRL/lpr), and C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). To generate Foxo3a-deficient mice, we utilized chimeric animals derived from the BayGenomics ES cell line XA026, obtained from the Mutant Mouse Regional Resource Center, University of California at Davis (Davis, CA). The mutant allele was backcrossed three times against the 129 background before intercross of heterozygotic animals to generate +/+ or trap/trap littermates for use in this study. Genotype was determined by PCR on tail DNA using primers 5′-AGTAACGGGCTTCCTCTTTCTCCTGTC, 5′-CTCTCCAG CCCTAGAAATCCAAACTC, and INV86R, 5′-CACTCCAACCTCCG CAAACTC (BayGenomics). All mice were maintained under specific pathogen-free conditions, and all experiments were performed in compliance with the relevant laws and institutional guidelines, as overseen by the Animal Studies Committee of the Washington University School of Medicine.

Plasmids and Retroviruses

An IκBε promoter-reporter construct was constructed by PCR from C57BL/6 genomic DNA, using primers 5′-GGGGTACCACCCCAC CACCACCACCAACCTACAC and 5′-GAAGATCTCCGAGCGGCTT CATTACCTGCC, which produced a ~515 bp fragment corresponding to the putative IκBε promoter (−497 to +118, respective to the transcriptional start site), flanked by KpnI and BglII restriction sites. The amplicon was cloned into the KpnI-BglII sites of TK-luc (Szabo et al., 2000) and then confirmed by routine sequencing. To generate a Foxo3a expression plasmid, a BamHI-XhoI fragment containing the full-length human Foxo3a cDNA (IMAGE clone 4137370, ATCC, Manassas, VA) was directionally cloned into the BamHI-XhoI sites of pcDNA3 (Invitrogen, Carlsbad, CA). To generate a retroviral Foxo3a expression plasmid, the same Foxo3a cDNA fragment was directionally cloned into the BamHI-XhoI sites of pMX-IRES-GFP (Lin et al., 2004; Morita et al., 2000; Onishi et al., 1996). pCMV-SPORT6-derived Foxm1- and Foxq1-expression plasmids were obtained from the ATCC (IMAGE clones 3881055 and 6489152, respectively). pCMV-SPORT6c (control pCMV-SPORT6) was generated by excising the SalI-NotI fragment containing the Foxm1 cDNA from pCMV-SPORT6-Foxm1. Retroviral supernatants were prepared in PlatE cells, and T and 293T cell transductions were performed as described (Lin et al., 2004; Morita et al., 2000; Onishi et al., 1996).

Luciferase Studies

Reporter assays were performed essentially as described (Lin et al., 2004). Briefly, for assays in M12 murine B cell lymphoma cells, 10^7 cells in 400 μl complete RPMI medium were electroporated in a 0.4 cm cuvette at 280 mV, 975 μF in the presence of 10 μg 2× NF-κB-luc, 40 ng pRL-CMV (*Renilla* luciferase control reporter, Promega, Madison, WI), and 10 μg pCDNA3 (Invitrogen), pcDNA3-Foxj1 (Lin et al., 2004), pcDNA3-Foxo3a, pCMV-SPORT6c, pCMV-SPORT6-Foxm1, or pCMV-SPORT6-Foxq1, and then returned to cell culture medium. After 4 hr, reporter activity was determined by the Dual-Luciferase Reporter Assay System (Promega) and relative activity determined after normalization for *Renilla* luciferase. For the 293T transformed human embryonic kidney line, 0.1–0.2 × 10^6 adherent cells in DMEM medium with 10% fetal calf serum were

transfected with 2 μg NF-κB-luc, 10 ng pRL-CMV, and 2 μg pcDNA3, pcDNA3-Foxj1, pcDNA-Foxo3a, pCMV-SPORT6c, pCMV-SPORT6-Foxm1, or pCMV-SPORT6-Foxq1, using FuGENE 6 (Roche). 24 hr later, cells were stimulated with 20 ng/ml recombinant human TNF-α (PeproTech), and 4 hr thereafter cells were analyzed via the Dual-Luciferase assay, with relative activity determined after normalization for *Renilla*. For primary T cells, 2×10^7 purified naive $CD4^+$ cells were electroporated with 20 μg NF-κB-luc, NF-AT-luc, or 6XDBE-luc (a Foxo reporter; Furuyama et al., 2000), as indicated, as well as 0.4 μg of pRL-CMV, with relative activity determined after normalization for *Renilla*.

Flow Cytometry

Flow cytometric analyses were performed on a FACSCalibur System (BD Biosciences, San Diego, CA) using splenocytes cleared of red blood cells by osmotic lysis or lymph node cells. Antibodies used included: FITC-16A (anti-CD45RB), APC-53-7.3 (anti-CD5), FITC-7D4 (anti-CD25), PE-IM7 (anti-CD44), PE-MEL-14 (anti-CD62L), CyChrome-RA3-6B2 (anti-CD45R/B220), PE-R6-60.2 (anti-IgM), and CyChrome-RM4-4 (anti-CD4; BD Pharmingen, San Diego, CA).

Lymphocyte Cultures and In Vitro Differentiation

For bulk $CD4^+$ Th cell analyses, splenocytes were cleared of erythrocytes by osmotic lysis, and $CD4^+$ cells purified by positive magnetic bead selection (Miltenyi Biotec, Auburn, CA). For naive-enriched $CD4^+$ Th cell analyses, lymph node cells from cervical, axillary, brachial, inguinal, and popliteal nodes were first cleared of $CD8^+$, $MHCII^+$, and $CD44^+$ cells by negative magnetic bead selection (Miltenyi Biotec), followed by positive $CD4^+$ magnetic selection. In general, cells were then incubated in complete RPMI medium supplemented with 10% fetal calf serum (BioWhittaker, Walkersville, MD), 10 mM HEPES, 1 mM sodium pyruvate, 2 mM glutamine, 50 μM β-mercaptoethanol, and 100 U penicillin/streptomycin (Sigma-Aldrich Chemical Co., St. Louis, MO) in 96-well U-bottom plates precoated with anti-CD3 (145-2C11, Pharmingen) at the concentrations indicated at 5×10^4 cells/well. Where indicated, 1 μg/ml soluble anti-CD28 (37.51, Pharmingen) and/or 100 U/ml recombinant human IL-2 (PeproTech, Inc, Rocky Hill, NJ) were added. For Th-neutral primary stimulation, Th cells were incubated in 12-well tissue culture plates at 0.5×10^6 cells/ml with 1 μg/ml plate bound anti-CD3 and 1 μg/ml soluble anti-CD28. For Th1 conditions, cultures were supplemented with recombinant murine IL-12 10 ng/ml (PeproTech) and 10 μg/ml anti-IL-4 (11B11, Pharmingen); for Th2 conditions, cultures were supplemented with 10 ng/ml recombinant murine IL-4 (PeproTech), 10 μg/ml anti-IFN-γ (XMG1.2), and 10 μg/ml anti-IL-12 (C17.8, Pharmingen). On day 3–4, T cells were expanded in complete medium containing 100 U/ml IL-2 and restimulated on day 6 with 1 μg/ml plate bound anti-CD3. Where indicated, anti-IL-2 (SB46, Pharmingen) was added at 10 μg/ml; phosphorothioate decoy or control decoy annealed NF-κB oligonucleotides, which inhibit the activity of all NF-κB subunits, were added at 10 μM (Khaled et al., 1998). Culture supernatants were assayed for IL-2, IL-4, IL-5, IL-6, IL-10, and/or IFN-γ by ELISA (Pharmingen) after 72 hr for primary stimulations, or 24 hr for secondary stimulations.

T Cell Proliferation Assays

Proliferation was assessed by 5-bromo-2′-deoxy-uridine incorporation (BrdU Labeling and Detection Kit III, Roche Molecular Biochemicals, Mannheim, Germany) on day 3 of T cell stimulation after 3–4 hr of cell labeling. For autologous mixed lymphocyte reactions, CD4 cells were stimulated under Th-neutral conditions, followed by expansion in IL-2, as above. Antigen-presenting cells were prepared from syngeneic splenocytes, irradiated by 30 Gy, and combined with day 6 primarily stimulated T cells at a 1:1 ratio in complete medium, 5×10^4 cells per well in a 96-well flat-bottom plate. Where indicated, concanavalin A (Calbiochem) was supplemented at 5 μg/ml. Proliferation was assessed by BrdU incorporation as above on day 3.

Immunohistochemistry

To determine NF-κB localization, 293T cells were plated at 0.1×10^6 cells per well of a 6-well tissue culture plate, each well containing a cover slip. After growth for 16–20 hr, the cells were infected with fresh retroviral supernatants of pMX-IRES-GFP versus pMX-Foxo3a-IRES-GFP viruses. 24 hours later, cells were treated with or without 20 ng/ml TNF-α for 45 min prior to fixation in 100% methanol (Sigma) for 5 min, −20°C. The coverslips were then washed thrice with PBS, blocked with 10% normal goat serum in PBS, incubated with primary antibody (C20, rat anti-RELA, Santa Cruz Biotechnology) at 1:100 dilution in PBS for 60 min in a humidified chamber, washed thrice with PBS, incubated with PE goat anti-rabbit IgG (Southern Biotechnology) at 1:100 dilution in PBS for 45 min, mounted on glass slides, and visualized by fluorescence microscopy. Nuclear versus cytoplasmic staining of NF-κB proteins was determined in GFP-positive cells.

Western Blotting

Total cell lysates were resolved by 7.5% SDS-PAGE electrophoresis and blotted to nitrocellulose. Membranes were blocked with 5% nonfat dried milk (Sigma), incubated with primary antibody at 1:200 dilution for 1 hr, washed thrice with PBS containing 0.05% Tween-20 (Sigma), incubated with HRP-conjugated mouse anti-goat or donkey anti-rabbit IgG (Pierce, Rockford, IL) at 1:5000 dilution for 1 hr, washed thrice with PBS-Tween, and then developed using ECL Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ) and BioMax MR film (Eastman Kodak Co., Chicago, IL). Primary antibodies included: polyclonal anti-whole Foxo3a (FKHRL1, Upstate, Lake Placid, NY) and polyclonal anti-Foxo1 (FKHR, Santa Cruz).

Histopathology

Tissue histology was performed on buffered formalin-fixed, paraffin-imbedded specimens with routine hematoxylin and eosin staining.

Immunoglobulin Studies

Chimeric animals were assayed for serum immunoglobulin titers and/or autoantibodies 4–6 weeks after chimerization by standard ELISA (Southern Biotechnology Associates, Birmingham, AL) and/or immunofluorescence protocols, as described (Peng et al., 2001, 2002).

RNA Transcript Analysis

For RNA analyses, RNA was prepared from cells at the times indicated in the text with the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA), and first-strand cDNA synthesized using oligo(dT) primers and SuperScript II reverse transcriptase (Invitrogen). Samples were then subjected to real-time PCR analysis on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) under standard conditions with specificity reinforced via the dissociation protocol. Foxo3a primers included 5′-CCTACTTCAAGGA TAAGGGCGA and 5′-GGTTGTGCCGGATGGAGTT. NF-κB, IκB, and Foxj1 primers have been previously described (Lin et al., 2004). Relative mRNA abundance of each transcript was normalized against tubulin (Gerth et al., 2003), calculated as $2^{(Ct[tubulin] - Ct[gene])}$, where Ct represents the threshold cycle for each transcript.

Apoptosis Studies

Activation-induced cell death was assessed by propidium iodide staining of purified T cell populations restimulated with $IL\text{-}2^{+/-}$ anti-CD3 or anti-CD95, after 2 days of primary stimulation with plate bound anti-CD3 and anti-CD28, as described (Refaeli et al., 2002). For IL-2-withdrawal-induced apoptosis, T cells were activated with plate bound anti-CD3, anti-CD28, and IL-2 for 3 days, washed extensively, and then resuspended in culture medium in the presence or absence of 100 U/ml IL-2. After 24 hr, apoptotic cells were identified by propidium iodide staining. For apoptosis in response to anti-CD3 in vivo, animals were injected intraperitoneally with 20 μg of a control (hamster IgG, Pierce) or anti-CD3 (145-2C11) antibody, as described (Bouillet et al., 2002). After 48 hr, thymi were harvested and analyzed by flow cytometry for the percentages of $CD4^+CD8^+$ thymocytes. For CD95-mediated apoptosis in vivo, female animals were injected intraperitoneally with 50 μg of a control hamster IgG or anti-CD95 (Jo2, Pharmingen) antibody, as described (Ogasawara et al., 1993). Survival was observed at 15–20 min intervals. For apoptosis effects in vitro, $CD4^+$ T cells were incubated in culture medium with 20 ng/ml anti-CD95 (Jo2), 2 ng/ml phorbol 12-myristate 13-acetate, or 1 μg/ml

ionomycin, as described (Bouillet et al., 1999). Viable cell count was determined daily thereafter.

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